

immunoprecipitated 5-lipoxygenase is quantitated by scanning densitometry.

[0233] A predicted result from these experiments would be as follows. The amount of 5-lipoxygenase protein immunoprecipitated from control cells would be normalized to 100%. Treatment of the cells with 1 μ M, 10 μ M, and 30 μ M of the macromolecules of the invention for 48 hours would reduce immunoprecipitated 5-lipoxygenase by 5%, 25% and 75% of control, respectively.

[0234] Measurement of 5-lipoxygenase enzyme activity in cellular homogenates could also be used to quantitate the amount of enzyme present which is capable of synthesizing leukotrienes. A radiometric assay has now been developed for quantitating 5-lipoxygenase enzyme activity in cell homogenates using reverse phase HPLC. Cells are broken by sonication in a buffer containing protease inhibitors and EDTA. The cell homogenate is centrifuged at 10,000 x g for 30 min and the supernatants analyzed for 5-lipoxygenase activity. Cytosolic proteins are incubated with 10 μ M 14 C-arachidonic acid, 2mM ATP, 50 μ M free calcium, 100 μ g/mL phosphatidylcholine, and 50mM bis-Tris buffer, pH 7.0, for 5 min at 37 °C. The reactions are quenched by the addition of an equal volume of acetone and the fatty acids extracted with ethyl acetate. The substrate and reaction products are separated by reverse phase HPLC on a Novapak C18 column (Waters Inc., Millford, MA). Radioactive peaks are detected by a Beckman model 171 radiochromatography detector. The amount of arachidonic acid converted into di-HETE's and mono-HETE's is used as a measure of 5-lipoxygenase activity.

[0235] A predicted result for treatment of DMSO differentiated HL-60 cells for 72 hours with effective the macromolecules of the invention at 1 μ M, 10 μ M, and 30 μ M would be as follows. Control cells oxidize 200 pmol arachidonic acid/5 min/ 10^6 cells. Cells treated with 1 μ M, 10 μ M, and 30 μ M of an effective oligonucleotide would oxidize 195 pmol, 140 pmol, and 60 pmol of arachidonic acid/5 min/ 10^6 cells respectively.

[0236] A quantitative competitive enzyme linked immunosorbant assay (ELISA) for the measurement of total 5-lipoxygenase protein in cells has been developed. Human 5-lipoxygenase expressed in *E. coli* and purified by extraction, Q-Sepharose, hydroxyapatite, and

reverse phase HPLC is used as a standard and as the primary antigen to coat microtiter plates. Purified 5-lipoxygenase (25 ng) is bound to the microtiter plates overnight at 4 °C. The wells are blocked for 90 min with 5% goat serum diluted in 20mM Tris!HCL buffer, pH 7.4, in the presence of 150mM NaCl (TBS). Cell extracts (0.2% Triton X-100, 12,000 x g for 30 min.) or purified 5-lipoxygenase were incubated with a 1:4000 dilution of 5-lipoxygenase polyclonal antibody in a total volume of 100 µL in the microtiter wells for 90 min. The antibodies are prepared by immunizing rabbits with purified human recombinant 5-lipoxygenase. The wells are washed with TBS containing 0.05% tween 20 (TBST), then incubated with 100 µL of a 1:1000 dilution of peroxidase conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA) for 60 min at 25 °C. The wells are washed with TBST and the amount of peroxidase labeled second antibody determined by development with tetramethylbenzidine.

[0237] Predicted results from such an assay using a 30 mer oligonucleotide at 1 µM, 10 µM, and 30 µM would be 30 ng, 18 ng and 5 ng of 5-lipoxygenase per 10⁶ cells, respectively with untreated cells containing about 34 ng 5-lipoxygenase.

[0238] A net effect of inhibition of 5-lipoxygenase biosynthesis is a diminution in the quantities of leukotrienes released from stimulated cells. DMSO-differentiated HL-60 cells release leukotriene B4 upon stimulation with the calcium ionophore A23187. Leukotriene B4 released into the cell medium can be quantitated by radioimmunoassay using commercially available diagnostic kits (New England Nuclear, Boston, MA). Leukotriene B4 production can be detected in HL-60 cells 48 hours following addition of DMSO to differentiate the cells into a neutrophil-like cell. Cells (2 x 10⁵ cells/mL) will be treated with increasing concentrations of the macromolecule for 48-72 hours in the presence of 1.3% DMSO. The cells are washed and resuspended at a concentration of 2 x 10⁶ cell/mL in Dulbecco's phosphate buffered saline containing 1% delipidated bovine serum albumin. Cells are stimulated with 10 µM calcium ionophore A23187 for 15 min and the quantity of LTB4 produced from 5 x 10⁵ cell determined by radioimmunoassay as described by the manufacturer.

[0239] Using this assay the following results would likely be obtained with an oligonucleotide directed to the 5-LO mRNA. Cells will be treated for 72 hours with either 1 μ M, 10 μ M or 30 μ M of the macromolecule in the presence of 1.3% DMSO. The quantity of LTB₄ produced from 5 x 10⁵ cells would be expected to be about 75 pg, 50 pg, and 35 pg, respectively with untreated differentiated cells producing 75 pg LTB₄.

E. In Vivo Assay

[0240] Inhibition of the production of 5-lipoxygenase in the mouse can be demonstrated in accordance with the following protocol. Topical application of arachidonic acid results in the rapid production of leukotriene B₄, leukotriene C₄ and prostaglandin E₂ in the skin followed by edema and cellular infiltration. Certain inhibitors of 5-lipoxygenase have been known to exhibit activity in this assay. For the assay, 2 mg of arachidonic acid is applied to a mouse ear with the contralateral ear serving as a control. The polymorphonuclear cell infiltrate is assayed by myeloperoxidase activity in homogenates taken from a biopsy 1 hour following the administration of arachidonic acid. The edematous response is quantitated by measurement of ear thickness and wet weight of a punch biopsy. Measurement of leukotriene B₄ produced in biopsy specimens is performed as a direct measurement of 5-lipoxygenase activity in the tissue. Oligonucleotides will be applied topically to both ears 12 to 24 hours prior to administration of arachidonic acid to allow optimal activity of the compounds. Both ears are pretreated for 24 hours with either 0.1 μ mol, 0.3 μ mol, or 1.0 μ mol of the macromolecule prior to challenge with arachidonic acid. Values are expressed as the mean for three animals per concentration. Inhibition of polymorphonuclear cell infiltration for 0.1 μ mol, 0.3 μ mol, and 1 μ mol is expected to be about 10%, 75% and 92% of control activity, respectively. Inhibition of edema is expected to be about 3%, 58% and 90%, respectively while inhibition of leukotriene B₄ production would be expected to be about 15%, 79% and 99%, respectively.